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(54) Title: METHOD AND SYSTEM FOR DETECTING OLIGONUCLEOTIDES IN A SAMPLE (57) Abstract <p>The present invention provides a method and a system for the detection of a target oligonucleotide in a sample. The method comprises, in general, the steps of providing a sensor device having a sensing interface carrying capturing oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a first portion of the target oligonucleotide; providing verification oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a second portion of the target oligonucleotide, other than the first portion; contacting the sample with the sensing interface under conditions so as to allow the target oligonucleotides, if present in the sample, to hybridize to the capturing oligonucleotides; prior to contacting the sample with the sensing interface or thereafter, allowing the verification oligonucleotides to hybridize to the target oligonucleotides if present in the sample; and detecting the presence of the verification oligonucleotides on the sensing interface. The detection of the verification oligonucleotides being indicative to the presence of the target nucleotide in the sample.</p>		

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METHOD AND SYSTEM FOR DETECTING OLIGONUCLEOTIDES IN A SAMPLE

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FIELD OF THE INVENTION

The present invention relates to a method and system for the detection of oligonucleotides in a sample.

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PRIOR ART

The following is a list of prior art references which are relevant for a better understanding of the background of the invention:

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12. PCT Application No. WO 97/04314.

Acknowledgement of these references in the description below will be made by
5 indicating the number from the above list.

BACKGROUND OF THE INVENTION

The development of DNA-sensor devices attracts substantial recent research efforts directed to gene analysis, detection of genetic disorders, tissue matching and forensic applications. Optical detection of DNA was accomplished by the application
10 of fluorescence labeled oligonucleotides^(1,2) or by the use of surface plasmon resonance^(3,4). Electronic transduction of the formation of oligonucleotide complexes with a target DNA, and, particularly, in the quantitative assay of DNA is a major challenge of bioelectronics⁽⁵⁾. The organization of DNA-sensors requires the assembly of the sensing interface on a transducer, and the design of the appropriate electronic
15 output that signals the formation of the recognition complex with the target DNA-analyte on the transducer element. Electrochemical DNA sensors based on the electrostatic attraction of electroactive transition metal complexes or organic dyes to oligonucleotide-DNA ds-complexes, e.g. Co(bpy)₃³⁺, acridin or Hoechst 33258 were reported⁽⁶⁻¹⁰⁾. Microgravimetric quartz-crystal- microbalance, QCM⁽¹¹⁾ analyses were
20 also applied to sense the formation of complementary oligonucleotide-DNA complexes.

Two major difficulties are still encountered in the development of DNA sensors and relate to the sensitivity and specificity of the resulting sensing systems.

GENERAL DESCRIPTION OF THE INVENTION

25 It is an object of the invention to provide a method and system for detecting target oligonucleotides in a sample.

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The term "*detect*" or "*detection*" refers collectively to both a qualitative determination of the presence of the target oligonucleotide in the sample as well as at times for evaluation of the level of the target oligonucleotide in the sample.

In accordance with the first aspect of the invention there is provided a method
5 for detecting a target oligonucleotide in a sample, comprising:

- (a) providing a sensor device having a sensing interface carrying capturing oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a first portion of the target oligonucleotide;
- (b) providing verification oligonucleotides having each a nucleotide
10 sequence complementary in at least a portion thereof to a second portion of the target oligonucleotide, other than said first portion;
- (c) contacting the sample with the sensing interface under conditions which allow the target oligonucleotides, if present in the sample, to hybridize to the capturing oligonucleotides;
- 15 (d) prior to (c) or thereafter, allowing the verification oligonucleotides to hybridize to the target oligonucleotides if present in the sample; and
- (e) detecting the presence of said verification oligonucleotides on the sensing interface.

In accordance with another aspect, the present invention provides a system for
20 detecting a target oligonucleotide in a sample, comprising:

- (i) a sensor device having a sensing interface carrying capturing oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a first portion of the target oligonucleotides;
- (ii) verification oligonucleotides having each a nucleotide sequence
25 complementary in at least a portion thereof to a second portion of the target oligonucleotide, other than said first portion; and
- (iii) a combination comprising one or both of apparatus and reagents for detecting a verification oligonucleotide bound to the sensing interface.

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The sample may be a biological specimen or a fractionation product thereof containing the oligonucleotides; a biological specimen treated to free and solubilized oligonucleotides; a specimen treated in a manner so as to digest nucleotide sequence into smaller oligonucleotides; a sample of oligonucleotides obtained by a PCR
5 (Polymer Chain Reaction) process or any other oligonucleotide amplification process; etc.

In accordance with one embodiment, the present invention may be applied for a variety of genetic screening assays, such as, for example, screening intended to locate mutant genes.

10 In accordance with another embodiment, the invention may be applied for identifying pathogens in a sample.

There is a wide variety of assaying techniques available for detecting oligonucleotides which are based on hybridizing a probe oligonucleotide to the target oligonucleotide. Also known are assay techniques wherein a probe oligonucleotide is
15 bound to a solid support which hybridize and "fish out" the target oligonucleotide from a tested sample. The invention is however unique in that it makes use of a verification oligonucleotide which increases both specificity and sensitivity of the assay.

In accordance with the invention, the verification oligonucleotide serves as an
20 indicator for the presence of the target oligonucleotide in the sample. In other words, detection of an immobilized verification oligonucleotide on the surface is an indication that the target oligonucleotide is bound to the sensing surface and hence that it existed in the sample. In accordance with the invention there are thus two discrimination means to ensure specificity and sensitivity:

- 25 1. Hybridization of the target oligonucleotide to the capturing oligonucleotide on the sensing surface. The complementary sequence of the capturing oligonucleotide will typically, but not exclusively, comprise a number of oligonucleotides completing about one helix of the nucleotide strand, i.e. about twelve nucleotides. A complementary

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sequence of twelve oligonucleotides ensure on the one hand stable hybridization. On the other hand, a 12-mer oligonucleotide decreases the chance of binding to an incorrect oligonucleotide than a longer sequence. In the case the sample is a digested specimen of genomic DNA, or a fractionation product thereof comprising the oligonucleotides, there is some probability, which increases with the length of the capturing oligonucleotide, of binding to an incorrect oligonucleotide, namely an oligonucleotide other than the target oligonucleotide. This probability is lower, as aforesaid in the case of a shorter oligonucleotide. A sequence of about 12 nucleotides is preferred as it is optimal as far as ensuring binding stability, on the one hand, and reducing incorrect binding on the other hand. The invention is, however, not limited to such a capturing oligonucleotide.

2. Hybridization of the verification oligonucleotide to the target oligonucleotide.

These two independent binding events thus reduce the chance of false positive or false negative results.

The detection of the verification oligonucleotide on the sensing surface may be achieved by a number of means. In accordance with one embodiment of the invention, the sensor device comprises an electrochemical probe for electrical/ electrochemical measurements, e.g. for Faradaic impedance spectroscopy measurement or amperometric detection of the oligonucleotide. In addition, detection may also be carried out by a number of other electrochemical techniques known *per se* based on the control of interfacial electron transfer rates between the sensing interface and the surrounding medium. For this electrochemical embodiment of the invention, the sensing surface is formed on a conductive matrix on which the capturing oligonucleotides are bound. Such an electrically conducting matrix may for example be made or coated by a metal such as gold, platinum, silver or copper.

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In accordance with another embodiment of the invention, the sensing device is a quartz crystal microbalance (QCM) probe in which case the presence of the verification oligonucleotide on the sensing surface is based on measurement of changes in resonance frequency of the probe. Microgravimetric QCM techniques are known *per se*, and are described, for example, in PCT Application WO 97/04314⁽¹²⁾.

In accordance with one preferred embodiment of the invention, the verification oligonucleotide is conjugated to a recognition agent which specifically binds to a signal-amplifying agent. The signal-amplifying agent, according to this embodiment, comprises a recognition partner capable of specific binding to the recognition agent.

10 The recognition agent and the recognition partner constitute together a recognition couple. In accordance with another preferred embodiment of the invention the verification oligonucleotide is bound to or complexed directly with a signal-amplifying agent.

The recognition couple may, for example, be one of the couples selected from

15 the group of biotin-avidin or biotin-streptavidin, receptor-ligand, sugar-lectin, antibody-antigen (the term "*antibody*" should be understood as referring to a polyclonal or a polyclonal antibody, to a fraction of an antibody comprising the variable, antigen-biotin binding portion, etc.). The recognition agent may be one member of the aforementioned couples, while the recognition partner may then be the

20 other member of the recognition couple.

In accordance with one embodiment of the invention, the verification oligonucleotide comprises a first recognition agent and the signal-amplifying agent comprises a second recognition agent, with the first and the second recognition agents being the same or different, and both being capable of specific binding to a

25 recognition partner to form a recognition couple. The recognition partner is thus capable of specific binding to both the first and the second recognition agents and thus its introduction to a sensing surface to which the verification oligonucleotide has bound, will yield binding of the signal-amplifying agent to the sensing interface. An example of a recognition partner is avidin or streptavidin, with both the first and

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second recognition agents being biotin. In accordance with an embodiment of the invention, the signal-amplifying agent comprises a plurality of said second recognition agents and thereby, by a sequence of exposures of the sensing interface to said recognition partner and said signal-amplifying agent, a complex comprising two or
5 more signal amplifying agents bound to each verification oligonucleotide on the sensing interface, may thereby be obtained to yield an increased signal amplification.

The signal-amplifying agent, according to one embodiment of the invention is a moiety or particle which directly increases the mass immobilized on the sensing surface. The signal-amplifying agent may, for example, comprise molecules, a super
10 molecular structure, or particle, e.g. colloid particles, macromolecules, clusters or molecules, liposomes, etc. In addition, the signal-amplifying agent may also be conjugated to or complexed with a label including, but not limited to an enzyme label. In case of an enzyme label, the enzyme is of a kind that can catalyze a reaction giving rise to an insoluble product. In accordance with this embodiment, the enzyme, after
15 the signal-amplifying agent binds to the recognition agent, is allowed to catalyze a reaction which gives rise to the insoluble product, and the product then precipitates onto the sensing surface. This product may then be detected by a variety of electric-electronic or optical detection means. In an assay carried out in accordance with the electrochemical embodiment, such a precipitate is preferably detected by the
20 large change in electrode impedance resulting therefrom or, alternatively, it may be detected by the mass change on a piezoelectric crystal resulting in a frequency change of the crystal.

In accordance with an embodiment of the invention, a particle, serving as a single-amplifying agent by its own right, may also carry an enzyme for further
25 amplification of the binding-associated signal. For example, a liposome used as a signal-amplification agent may be bound to or complexed with said enzyme to allow further increase in mass as a result of precipitation of the enzyme-catalyzed insoluble product on the sensing surface, and thus a further amplified binding- related signal.

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The invention also provides, for use in the above method and system, one or more reagents, selected from the group consisting of:

- (i) said verification oligonucleotide;
- (ii) an amplifying agent for amplifying the signal resulting from binding
5 of said verification oligonucleotide to said sensing interface.

The invention will now be described with reference to a non-limiting specific embodiment. As will no doubt be appreciated, this description is a mere illustrative example of the wider scope of the invention as defined in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 illustrates a bioelectronic system in accordance with an embodiment of the invention including assembly of the sensor and its use in detection of an target oligonucleotide in a sample.

Figs. 2A-2D illustrate in somewhat more details some of the components of the system of Fig. 1 as used in the exemplary experiments: Fig. 2A shows a DNA strand
15 covalently bound to biotin; Fig. 2B illustrates the chemical reaction, catalyzed by horseradish peroxidase (HRP), in which 4-chloro- naphthol is reacted to form an insoluble product; Fig. 2C shows the structure of the thiophosphate thymine; and Fig. 2D shows the sequence of some oligonucleotides used in the exemplary experiments.

Figs. 3A and 3B show the impedance features, presented as Nyquist plots, of a
20 bare electrode (curve a), after functionalization of the electrode with the capturing oligonucleotide (curve b), after binding the target DNA and the biotinylated oligonucleotide hybrid (curve c), after interaction with the avidin-HRP conjugate (curve d) and after some period of catalysis of the enzyme resulting in deposit of insoluble product on the sensing surface (curve e). It should be noted that Figs. 3A
25 and 3B are of the same experiment but drawn to different scales.

Fig. 4A illustrates a bioelectronic system in accordance with a further embodiment of the invention including assembly of the sensor and its use in detection of a target oligonucleotide in a sample.

Fig. 4B shows the sequence of the different oligonucleotides used in a system of the kind illustrated in Fig. 4B, in the accompanying experiments.

Figs. 5A shows the impedance spectra presented as Nyquist plots of the feature illustrated in Fig. 4, the spectra including: a functionalized electrode carrying the capturing oligonucleotides (curve a); after interacting the functionalized electrode with a sample carrying the target oligonucleotide (curve b); after interaction with the oligonucleotide-functionalized liposome (curve c); after interacting an electrode functionalized with a mutated capturing oligonucleotide (curve d); and after treatment of the mutated capturing oligonucleotide bearing electrode with functionalized liposome (curve e).

Fig. 5B shows the changes in electron transfer resistance of a functionalized electrode upon treatment with different concentrations of the target oligonucleotide and amplification with labeled liposomes.

Fig. 6 illustrates a bioelectronic system in accordance with a yet further embodiment of the invention including assembly of the sensor and its use in detection of a target oligonucleotide in a sample.

Fig. 7A shows the impedance spectra presented as Nyquist plots of the features illustrated in Fig. 6, the spectra including: a functionalized electrode (curve a); after interaction with a sample carrying the target oligonucleotide pre-treated with a biotin-labeled verification oligonucleotide (curve b); after subsequent interaction with avidin (curve c); after subsequent interaction with biotinylated liposomes (curve d); after subsequent interaction for a second time with avidin (curve e); and after a subsequent interaction for a second time with the biotinylated liposomes (curve f).

Fig. 7B shows a calibration curve which corresponds to the changes in the electron transfer resistances of the functionalized electrode upon interaction with different concentrations of the target oligonucleotide and enhancement of the detecting processes by a double-step avidin/biotinylated liposome amplification path.

Fig. 8A shows time-dependent frequency changes of oligonucleotide-bound crystal after interaction with a sample containing the target oligonucleotide (curve a);

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after interaction of the resulting electrode with an oligonucleotide-labeled liposome (curve b); after functionalizing a crystal with a mutated capturing oligonucleotide (curve c); after bringing into contact the mutated oligonucleotide-labeled crystal with oligonucleotide-labeled liposome (curve d); after treating an oligonucleotide-labeled
5 crystal with oligonucleotide-labeled liposomes (curve e).

Fig. 8B shows time-dependent frequency changes of oligonucleotide-labeled crystal after interacting with a sample containing the target oligonucleotide (Curve e); after interacting the resulting interface with avidin (curve f); after interacting the resulting assembly with biotinylated liposome (curve h); after further interacting with
10 avidin (curve g); after further interacting with biotinylated liposome (curve i); after treating an oligonucleotide labeled crystal with a mutated oligonucleotide pre-treated with biotinylated liposomes (curve j); after treating the resulting interface (of curve j) with avidin (curve k); and after treating with biotinylated liposomes.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

15 The manner of assembly of the DNA sensor in accordance with an embodiment of the invention and its use, are illustrated in Fig. 1. Oligonucleotide 100, serving as the capturing oligonucleotide, includes a first portion 102, typically a 12-base sequence, that is complementary to a first portion of the target oligonucleotide, and a second portion 106 for binding to the electrode, e.g. a gold (Au) electrode 108. The
20 binding portion 106 may, for example, be a several base (e.g 5) thiosphosphate thymidine (TS) sequence, illustrated in Fig. 2C. Occasionally the two portions 102, 106 may be separated by one or more separator base-nucleotides. The electrode 108 and oligonucleotide 100 are reacted such that portion 106 binds to the surface of the Au electrode. As a result, functionalized electrode with a sensing surface 110 is
25 formed.

A verification oligonucleotide 112 is contacted with a sample which contains the target oligonucleotide 104 whereby a partial double-stranded structure 114 is formed. This structure is then contacted with the sensing surface (step B) yielding a

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bifunctional double-stranded oligonucleotide assembly 116. It should be noted that it is possible in accordance with another embodiment of the invention to first contact the sensing surface 110 with the sample and only then bring a reagent solution which comprises the verification oligonucleotide 112 into contact with the sensing surface.

5 This will first yield binding of the target oligonucleotide 104 (if present in the sample) to the sensing surface and then binding of verification oligonucleotide 112 to yield assembly 116. In both cases, the presence of the verification oligonucleotide 116 on the sensing surface serves as an indication of the presence of the target oligonucleotide 104 in the sample.

10 The detection of the verification oligonucleotide on the sensing surface may be achieved by a number of means some of which were explained above. For example, the verification oligonucleotide may carry a label which may be detected electrically, e.g. by determining change in impedance, or electron transport between the electrode 108 and the surrounding medium. The label, by one embodiment, is an enzyme which
15 can catalyze a reaction yielding an insoluble reaction product which precipitates on the surface's electrode thus increasing impedance. This is illustrated in step C of Fig. 1.

In accordance with one embodiment, verification oligonucleotide 112 is bound to a biotin moiety 117. A label complex 118 which comprises an avidin 119 bound to
20 an enzyme 120 is contacted with the sensing surface (step C) resulting in binding of complex 118 to the sensing surface. Enzyme 120 can catalyze a reaction converting a substrate (S) into an insoluble product (P) which is thus deposited on the sensing surface. Both the binding of the labeling complex 118 to the sensing surface as well as the precipitation of product (P) onto the sensing surface can be monitored similarly as
25 above (i.e. change of impedance or a change of mass in the case of QCM-type measurement).

In Figs. 4A, 4B and 6, the same reference numerals as used in Fig. 1 are used for like components.

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According to another embodiment illustrated in Fig. 4A, use is made with a verification oligonucleotide-modified liposome 134 that is bound to the double-stranded immobilized assembly 132, to form an immobilized double-stranded oligonucleotide-liposome assembly 138. The binding of the labeled verification liposome onto the sensing surface can be monitored as described above.

The sensing of the target oligonucleotide 104 in accordance with a further embodiment, can be further amplified by using a double-step avidin/biotin-labeled-liposome amplification pathway as shown schematically in Fig. 6. Functionalized electrode 110 is first hybridized with target DNA 104 pre-treated with biotin-labeled oligonucleotide 112 having a portion sequence complementary with oligonucleotide 100, immobilized on said electrode 108, to form bifunctional double-stranded biotinylated assembly 116. The formed assembly is then reacted with biotinylated liposomes 142 to form a liposome containing assembly 144. This assembly can further be reacted with avidin and additional biotinylated liposomes, to yield a multi-liposome assembly 146.

The invention will now be further illustrated by the following example:

EXAMPLES

For clarity, in the description below the same reference numeral to those used above will be used. However, by doing so, it should not, in any way, limit the scope of the invention to the specific examples below.

It should be noted that the scheme shown in Fig. 1 can be employed for various different assays than that specifically exemplified herein. Furthermore, a similar scheme, *mutatis mutandis*, may also be used for assaying a target oligonucleotide in other assay techniques, e.g. microgravimetric QCM. In this latter case rather than electric/electronic measurements, the measurement is of change in resonance frequency of the piezoelectric crystal as a result of mass change.

Example 1 Enzyme-amplified detection of a target oligonucleotide in a sample

The sensor preparation sequence as used in the Example can be seen in Fig. 1, while the sequences of the oligonucleotides used can be seen in Fig. 2D. In Fig. 2D each oligonucleotide is identified by the reference numerals used in the example.

5 An 18-mer oligonucleotide 100 (SEQ ID NO: 1) which included a 12-base sequence 102 that is complementary to a part of the analyte, the Tay-Sachs (TS) mutant 104 (SEQ ID NO: 2) was used. In addition, oligonucleotide 100 included a 5-base thiophosphate thymine-TS tag 106 for its assembly on the gold (Au) electrode 108, and a single T-base separating the tag from the sensing oligonucleotide
10 sequence. A disc Au-electrode 108, 0.05 cm^2 , was interacted with oligonucleotide 100 ($20 \text{ } \mu\text{M}$, 10 hours) resulting in the assembly of the sensing interface on the gold support (step A in Fig. 1). The resulting functionalized electrode 110 was interacted with a solution that included the target analyte, the TS-mutant sequence. 104 ($5.8 \times 10^{-7} \text{ g/mL}^{-1}$, 4 hours), and a biotinylated verification oligonucleotide 112 (SEQ ID
15 NO: 3, bound via the 5' end to biotin, Fig. 2D), $2 \times 10^{-5} \text{ g/mL}^{-1}$ (step B in Fig. 1).

Verification oligonucleotide 112 is complementary to one portion of an oligonucleotide 104 and consequently these two oligonucleotides hybridize to form a partial double-stranded structure 114. Target oligonucleotide 104 has another sequence complementary to portion 102 of capturing oligonucleotide 100 and thus step B results
20 in the formation of a bifunctional double-stranded DNA-oligonucleotide assembly 116.

Sensing surface with bifunctional double-stranded DNA- oligonucleotide assembly 116 is then treated with an avidin labeled with horseradish peroxidase (HRP) ($1 \times 10^{-8} \text{ g/mL}^{-1}$, 3 hours) (step C in Fig. 1). HRP can catalyze the oxidation of
25 4-chloro-1-naphthol (S) by hydrogen peroxide (H_2O_2) giving rise to the formation of an insoluble product (P) which precipitates on the electrode. Other enzyme-substrate couples yielding an insoluble product which may be used include: alkaline

phosphatase and indoyl phosphate derivatives as substrates; glucose oxidase and tetrazolium salts as substrates; etc.

As the oligonucleotide and oligonucleotide-DNA layered assemblies are negatively charged, the electrostatic repulsion of a negatively-charged redox-probe, e.g. $\text{Fe}(\text{CN})_6^{3-/4-}$, from the electrode support is anticipated to perturb the interfacial electron transfer. This is expected to introduce an electron transfer resistance that can be detected by Faradaic impedance spectroscopy or other electrochemical means such as reduction of the amperometric response of the electrode. The biocatalytic precipitation of the product (P) on the electrode is expected to further insulate the conductive support and to lead to a high interfacial electron transfer resistance or a reduction of the amperometric response of an electroactive species solubilized in the medium surrounding the electrode.

Fig. 3A shows the impedance features, using $\text{Fe}(\text{CN})_6^{3-/4-}$ as redox-probe, presented as Nyquist plots (Z_{im} vs. Z_{re}), of the bare electrode 108 (curve a), of the functionalized electrode with the sensing surface 110 (curve b) and of the layered bifunctional double-stranded oligonucleotide-target DNA and biotinylated oligonucleotide assembly (curve c). The respective semicircle diameters correspond to the interfacial electron transfer resistances, R_{et} . It can be seen that the electron transfer resistance increases upon the build-up of the biotinylated oligonucleotide-DNA assembly. For example, for the functionalized electrode $R_{\text{et}} = 1.1 \text{ k}\Omega$ whereas R_{et} is increased to about $2.2 \text{ k}\Omega$ upon the association of the complex 114. These results are consistent with the fact that the negative charge increases upon the two-step organization of the assembly. This results in the enhanced electrostatic repulsion of the redox-probe, and introduces higher interfacial electron transfer resistance.

Fig. 3B shows the impedance spectra of the bifunctional double-stranded assembly consisting of the target DNA linked to the sensing interface and the biotinylated oligonucleotide, before (curve c) and after (curve d) interaction with the avidin-HRP conjugate. Upon the association of the avidin-HRP biocatalytic conjugate to the layer, a considerable increase in the electron transfer resistance is observed due

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to the partial insulation of the electrode by the proteins. In the presence of H_2O_2 and the substrate (S), the biocatalytic precipitation of the product onto the electrode occurs. This insulates the conductive support, resulting in a very high increase in the electron transfer resistance, curve (e), $R_{et} = 17 \text{ k}\Omega$. It should be noted that the two
5 parameters controlling the sensitivity of the DNA-sensing devices are the time of incubation of the functionalized-monolayer-electrode 110 with the complex 114 and more important, the time-interval used to precipitate the product by the avidin-HRP biocatalytic conjugate. Using this configuration, and upon precipitation of P for 40 min. it was possible to sense the target DNA 104 at a concentration of $20 \times 10^{-9} \text{ g/mL}^{-1}$, $R_{et} = 7.9 \text{ k}\Omega$.
10

Control experiments show that the oligonucleotide sensing assembly has a high specificity and selectivity. Treatment of the functionalized electrode 110 with the biotinylated oligonucleotide 112 and then with the avidin-HRP conjugate 118, but without the interaction with the target DNA 104, yielded only a minute change in the
15 electron transfer resistance.

In order to test the specificity of the system, the same assay was performed with a DNA fragment 104' (SEQ ID NO: 4) that corresponds to the normal gene sequence in which the 7-based mutation leads to the TS-genetic disorder. After contact of the sensing interface with a complex between fragment 104' and the verification
20 oligonucleotide 112, the system was subjected to the biocatalytic precipitation process using the avidin-HRP conjugate, using the same protocol as illustrated in Fig. 1. However, no noticeable changes in the electron's transfer resistance at the electrodes were observed, implying that the lack of formation of a complex between the capturing oligonucleotide on the sensing surface and the complex formed between the
25 target oligonucleotide 104 and the verification oligonucleotide 112 which prevented the subsequent formation of the precipitant layer on the electrode.

Cyclic voltammetry experiments (see insert Fig. 3B) further confirm the stepwise organization of the bifunctional double-stranded complex 116, and that the precipitation of the insulating layer formed by product P on the electrode, gradually

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perturb the electron-transfer kinetics of $\text{Fe}(\text{CN})_6^{3-}$. Fig. 3B inset, shows the cyclic voltammograms of $\text{Fe}(\text{CN})_6^{3-}$ at a bare Au- electrode (curve a), upon formation of the sensing assembly 110 (curve b), and upon the formation of the double-stranded assembly 110 (curve c). The stepwise assembly of the layers is accompanied by a decrease in the amperometric response of the electrode and an increase in the peak-to-peak separation between the cathodic and anodic waves of the redox-probe. This is consistent with the enhanced electron transfer barriers introduced upon the assembly of the negatively-charged oligonucleotide assembly. Association of the avidin-HRP conjugate onto the layer (curve d), further separates the redox waves of $\text{Fe}(\text{CN})_6^{3-}$ implying that binding of the protein insulates the electrode and perturbs the interfacial electron transfer. Biocatalytic precipitation of P onto the electrode insulates the conductive support, and the electrical response of the redox-probe is almost entirely blocked, (curve e). The result shown in the inset of Fig. 3B demonstrates that amperometric transduction of the formation of the complex 116, binding of avidin-linked HRP 118, and further precipitation of the product P is possible.

By some modification of the assayed scheme described above, rather than determining the formation of the insoluble product precipitates on the electrode by means of a Faradaic impedance spectroscopy, it may also be determined by means of amperometric detection, by optical means and others or by microgravimetric QCM detection, an example for the latter being provided hereinbelow.

Example 2 Liposome-amplified detection of an oligonucleotide

The sensor preparation sequence as used in the example can be seen in Fig. 4A, while the sequence of the oligonucleotides used can be seen in Fig. 4B. In Fig. 4B, each oligonucleotide is identified by the reference numeral as used in the examples.

A mercaptohexyl oligonucleotide 100 (SEQ ID NO: 5 bound to the mercaptohexyl via the 3' end) including a portion 102 that is complementary to a part of the analyte 104 (SEQ ID NO: 6) and the mercapto-derived portion 106 for its assembly as a monolayer on an Au-electrode 108 was used as a capturing agent. The

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mercaptohexyl oligonucleotide 100 was assembled on the Au-electrode 108 as a monolayer, to obtain the sensing interface 110 (step A in Fig. 4A). A surface coverage of the electrode of 1.1×10^{-11} mole/cm² was determined by Tarlov's electrochemical method [Tarlov M.J. *et al.* Anal. Chem. 70:4670 (1998)], and comparable results were
5 obtained by QCM analyses. The resulting monolayer-functionalized electrode 110 was then brought into contact with a sample containing the target analyte, oligonucleotide 104 (5×10^{-6} M, 15 hours incubation, 25°C), to yield a double-stranded assembly 132 (step B in Fig. 4A) wherein at least part (130) of the assembled analyte is left free for further hybridization. The resulting electrode interface was then treated with
10 oligonucleotide-labeled liposome 134 (lipid concentration 0.2mM, 15 min. 25°C). The oligonucleotide moiety 136 (SEQ ID NO: 7, bound to a mercaptohexyl group via the 3' end, Fig. 4B) within the labeled liposome 134 is complementary to the residual base-sequence 130 of the analyte. Thus, a liposome-linked three-component double-stranded assembly 138, consisting of the capturing agent 100, the analyte 104,
15 and the liposome tagged with oligonucleotide 136, is generated on the electrode support.

The oligonucleotide-labeled-liposome was prepared by the assembly of liposomes that are composed of phosphatidic acid, phosphatidyl choline, maleimide-phosphatidylethanolamine, cholesterol (marked with 3H-cholesterol, 45
20 Ci/mole) at a ratio of 79:20:1:0.1, that were modified with oligonucleotide 136 by incubation therewith for 20 hours at 4°C and purified by chromatography (Sephadex G-75). The surface coverage of the liposome with oligonucleotide 136 (50-60 oligonucleotide units per liposome) was determined by reacting the resulting liposomes with Oligreen (Molecular probe) and following fluorescence intensity of
25 the resulting liposome suspension at $\lambda=480\text{nm}$. The size of the liposomes was determined by dynamics light-scattering and corresponded to 220 ± 20 nm.

The oligonucleotide-labeled liposomes 134 are negatively charged in order to eliminate non-specific adsorption of the liposomes onto the sensing interface. The liposomes associated with the electrode support represent "giant" negatively charged

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amplifying agents that electrostatically repel a negatively charged redox-probe stabilized in the electrolyte solution. That is, the biorecognition event between the capturing oligonucleotide 100 and the target oligonucleotide 104 is amplified by the generation of a highly-charged microenvironment that repels the electroactive probe, $\text{Fe}(\text{CN})_6^{3-/4-}$, in solution. The electron transfer resistance produced by the assembly 138 was then assayed by Faradaic impedance spectroscopy.

Fig. 5A shows the impedance spectra (in the form of Nyquist plots, Z_{im} vs. Z_{re}) of oligonucleotide-functionalized electrode 110 (curve a) after hybridization with the target oligonucleotide 100 to form the layered double-stranded oligonucleotide assembly 132 (curve b), and after interaction with the probing oligonucleotide-labeled liposome 136 to form the amplified assembly 138 (curve c). While a bare Au-electrode exhibits an electron transfer resistance of 0.5 k Ω , the associated of the capturing oligonucleotide 100 onto the conducting support increased the electron transfer resistance to 3 k Ω . This is attributed to the electrostatic repulsion of the redox label, $\text{Fe}(\text{CN})_6^{3-/4-}$, that results in a barrier for the interfacial electron transfer. The formation of the double-stranded assembly with the target oligonucleotide increased the electron-transfer resistance to $R_{\text{et}}=4.5 \text{ k } \Omega$. This is consistent with the results presented by Example 1, hereinabove and with the fact that the higher negative charge formed on the surface as a result of hybridization, enhances the electrostatic repulsion of the electroactive species on the solution. Binding of the oligonucleotide-modified liposome 134 introduced a very high electron transfer resistance corresponding to 15 k Ω . This result is attributed to the formation of a negatively charged micro-interface upon the association of the liposome to the double-stranded assembly.

A control experiment, for the evaluation of the system's specificity, was conducted, which included an attempt to detect the presence of an oligonucleotide 104' (SEQ ID NO: 8), that included a 6-base mutation relative to the target DNA 104. Fig. 5A further shows the impedance spectrum of the functionalized-electrode 110 after its treatment with the mutant 104' (curve e) and the impedance spectrum of the resulting electrode after further treatment with the

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oligonucleotide-labeled liposome 134 (curve e). As may be understood from the results presented in Fig. 5A, the interfacial electron transfer resistances were almost unchanged in this control experiment, implying that the sensing interface is selective for analyses of target oligonucleotide 104. The results also indicate that no
5 non-specific association of mutant 104' or of the oligonucleotide-labeled liposomes 134 on the electrode took place. This is attributed to the electrostatic repulsion existing between these components and the sensing interface.

The extent of increase in the electron transfer resistance upon the binding of the analyte-oligonucleotide, and the secondary association of the modified liposome is
10 controlled by the bulk concentration of the analyte, as shown in Fig. 5B. the lower sensitivity limit for analyzing the analyte DNA was determined to be 1.2×10^{-12} M at a signal-to-noise value of $S/N = 3$.

A further control experiment, where only the oligonucleotide 136 interacted with the double-stranded assembly of the capturing oligonucleotide 100 and the target
15 oligonucleotide 104 introduced only a small increase in the electron transfer resistance, $R_{et} = 4.7 \text{ k}\Omega$., indicating that the negatively charged liposome indeed amplified the electrostatic repulsion of the redox label.

The sensing system may be further amplified as schematically illustrated in Fig. 6, wherein the presence of target oligonucleotide 104 (SEQ ID NO:1) was
20 detected using the negatively-charged liposomes 142 carrying the biotinylated oligonucleotide 136' (SEQ ID NO:9 bound via the 5' end to biotin). Accordingly, oligonucleotide-functionalized electrode 110 is reacted with the target oligonucleotide (5×10^{-6} M, 15 min. of hybridization, at 25°C), pre-treated with biotinylated verification oligonucleotide 112 (SEQ ID NO:5) 1×10^{-5} M, interaction time 2 hr.
25 25°C), being complementary to segment 102 of the target oligonucleotide (step A, in Fig. 6). This process results in a three-component double-stranded-assembly on the electrode, consisting of the capturing oligonucleotide 100, the analyte oligonucleotide 104 and the biotin-labeled oligonucleotide 112. Association of avidin 118 (8 min. of incubation, step B in Fig. 6) and then the biotin-tagged-liposome 142 (8 min. of

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incubation, step C in Fig. 6) resulted in the formation of a negatively-charged interface 144 that amplified the primary oligonucleotide recognition event by the electrostatic repulsion of $\text{Fe}(\text{CN})_6^{3-/4-}$ and the enhancement of the interfacial electron transfer resistance. This sensing configuration enabled the further amplification of the biorecognition event by the multiple reaction of the resulting array with avidin and then with the biotinylated liposomes to yield a dense array of the negatively-charged liposomes. The biotin-labeled liposomes were composed of phosphatidyl choline, phosphatidylethanolamine, cholesterol (marked with ^3H -cholesterol, 45Ci/mole) and biotinylated phosphatidylethanolamine with a ratio corresponding to 80:20:0.1:0.1. The average coverage of the liposomes with biotin corresponded to 550, which were purified by gel chromatography (DEAE Sephadex A-25). The size of the liposomes was determined by dynamic light scattering to be 180 ± 40 nm.

Fig. 7A shows the impedance spectra of the array in the different steps of modification. The oligonucleotide-functionalized interface 108 exhibited an electron transfer resistance corresponding to $3 \text{ k}\Omega$ (curve a), and upon the formation of the double-stranded assembly with the analyte-DNA 104 complexed with the biotinylated oligonucleotide 112 to form immobilized biotinylated analyte 116, the electron transfer resistance increased to $R_{\text{et}} = 4.8 \text{ k}\Omega$. (curve b). Association of avidin 119 ($2.5 \mu\text{g/ml}$) to the interface 116 further increased the electron transfer resistance to $7.6 \text{ k}\Omega$, as a result of the hydrophobic, insulating features of the protein (curve c). Association of the biotin-labeled liposome to the sensing surface 30 min. lipid concentration 0.25 mM), substantially increased the electron transfer resistance, $R_{\text{et}} = 14.8 \text{ k}\Omega$. (curve d).

The sensing of the target-DNA was further amplified by the application of a second step of association of the avidin-biotinylated liposomes under the same conditions (step D in Fig. 6), that enhanced the electron transfer resistance, respectively, to $17 \text{ k}\Omega$ and $20 \text{ k}\Omega$ (curves e and f, in Fig. 7).

In a control experiment, the sensing interface was interacted with mutant, non-complementary DNA 104' (SEQ ID NO:4 , $5 \times 10^{-6} \text{ M}$), pre-treated with

biotinylated oligonucleotide 112 and subsequently treated with avidin and the biotinylated liposome, under the same conditions. A minute increase in the electron-transfer resistance corresponding to $R_{et} = 3.4 \text{ k}\Omega$ was observed, attributed to non-specific adsorption of avidin to the sensing interface.

- 5 The increase in the electron-transfer resistance at the electrode upon binding of avidin and the biotin-labeled liposome, were controlled by the bulk concentration of the target-DNA in the sample (Fig. 7B).

Using a double-step avidin/biotin-labeled-liposome amplification pathway, target DNA concentration as low as 5×10^{-14} (signal to noise ratio $S/N=3$) was
10 detected.

- In a similar manner to that described with reference to Fig. 4A, an oligonucleotide capturing agent was assembled on an Au/quartz crystal. The functionalized interface was then hybridized with a target DNA (concentration $5 \times 10^{-6} \text{ M}$) followed by interaction thereof with the oligonucleotide-labeled liposome.
15 Fig. 8A (solid line) shows QCM-transduction of the amplified sensing of the analyte. Interaction of the functionalized crystal with the analyte (point a) resulted in a frequency decrease of $\Delta f = 17 \text{ Hz}$, implying a surface coverage of the analyte corresponding to $1.2 \times 10^{-11} \text{ mole/cm}^2$. Further reaction of the double-stranded surface with the oligonucleotide-tagged liposome (point b) resulted in a substantial decrease
20 in the crystal frequency, $\Delta f = -120 \text{ Hz}$.

Fig. 8A shows also the results of a control experiment in which the sensing interface was interacted with the mutated, non-complementary oligonucleotide ($5 \times 10^{-6} \text{ M}$, point c) followed by treatment with the tagged liposome (point d). As shown, the crystal frequency was unchanged $\Delta f = \pm 2 \text{ Hz}$ upon interaction with the
25 non-complementary DNA. Interaction with the tagged liposome slightly altered the crystal frequency, $\Delta f = -5 \text{ Hz}$. This frequency change may be attributed to minute non-specific binding of the liposome to the interface. Association of the amplified oligonucleotide-tagged liposome with the interface resulted in a frequency change of $\Delta f = -70 \text{ Hz}$ (point e), that allows the easy amplified detection of the target DNA also

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by using microgravimetric QCM assay. The lowest sensitivity limit for the detection of the target DNA by this amplification method was estimated to be 5×10^{-12} M ($\Delta f = -20$ Hz, after treatment with tagged liposome).

Fig. 8B shows the results of sensing a target DNA in a sample the manner described in connection with Fig. 6, however also in this case, wherein the a capturing oligonucleotide is assembled on a Au/quartz crystal. Accordingly, first an analyte-double-stranded biotinylated system was associated with the sensing interface which resulted in a frequency decrease of 25 Hz (curve e). Binding of avidin to the biotinylated assembly yielded a frequency change of $\Delta f \sim 50$ Hz (point f). Linkage of the biotin-tagged liposomes to the system amplified the primary association of the analyte and a very high frequency change $\Delta f \sim 500$ Hz was observed (point g). Additional treatment of the interface with avidin, $\Delta f \sim 50$ Hz (point h) and then with the biotin-labeled liposome (point i) resulted in a second amplification corresponding to $\Delta f = 690$ Hz.

Treatment of the sensing interface with the biotin-labeled non-complementary DNA did not yield any significant frequency change (point j) and subsequent interaction of the resulting assembly with avidin and the biotin-tagged liposome resulted in a frequency change of about -30 Hz (points k and l, respectively). As described above, this change in frequency may be attributed to non-specific association of the liposome to the interface. Using the two-step amplification pathway, the lowest sensitivity limit for sensing the target DNA was estimated to be 1×10^{-13} M (or 1×10^{-16} mole/ml), which may be further enhanced by performing additional binding steps of avidin-biotinylated liposome.

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CLAIMS:

1. A method for detecting a target oligonucleotide in a sample, comprising:
 - (a) providing a sensor device having a sensing interface carrying capturing oligonucleotides having each a nucleotide sequence complementary in at least a
5 portion thereof to a first portion of the target oligonucleotides;
 - (b) providing verification oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a second portion of the target oligonucleotide, other than said first portion;
 - (c) contacting the sample with the sensing interface under conditions such
10 so as to allow the target oligonucleotides, if present in the sample, to hybridize to the capturing oligonucleotides;
 - (d) prior to (c) or thereafter, allowing the verification oligonucleotides to hybridize to the target oligonucleotides if present in the sample; and
 - (e) detecting the presence of said verification oligonucleotides on the
15 sensing interface.
2. The method of Claim 1, wherein said sensor device comprises an electrochemical probe carrying the sensing interface.
3. The method of Claim 2, wherein said detection is based on Faradaic impedance spectroscopy or amperometric measurements.
- 20 4. The method of Claim 1, wherein said sensor device comprises a microbalance quartz-crystal probe carrying the sensing interface.
5. The method of Claim 4, wherein said detection is based on a microgravimetric quartz-crystal microbalance (QCM) analysis.
6. The method of any one of Claims 1-5, wherein the sequence complementary
25 to at least a portion of the target oligonucleotide is of about 12 nucleotides.
7. The method according of any one of Claims 1-6, wherein the verification oligonucleotide is conjugated to a recognition agent which can specifically bind to a signal-amplifying agent, and step (e) of the method comprises:
 - (e1) contacting the sensing interface with said signal-amplifying agent;

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(e2) detecting the presence of said signal-amplifying agent on the sensing interface.

8. The method of Claim 7, wherein said recognition agent is biotin and said signal amplifying agent comprises avidin.

5 9. The method of any one of Claims 1-6, wherein said verification oligonucleotide is bound to or complexed with a signal-amplifying agent, and step (e) comprises detecting of presence of the signal-amplifying agent on the sensing interface.

10 10. The method of any one of Claims 1-6, wherein the verification oligonucleotide comprises a first recognition agent which specifically binds to a recognition partner to form a recognition couple, step (e) of the method comprises the following steps:

(e1) contacting said sensing interface with said recognition partner;

15 (e2) contacting said sensing interface with a signal-amplifying agent comprising a second recognition agent, which may be the same or different as the first recognition agent, which can also bind to said recognition partner; and

(e3) detecting presence of said signal-amplifying agent on said sensing interface.

20 11. The method of Claim 10, comprising the following step between steps (e2) and (e3):

(e2.1) repeating steps (e1) and (e2) one or more times.

12. The method of any one of Claims 7-11, wherein said signal-amplifying agent comprises an enzyme which catalyzes a reaction yielding an insoluble reaction
25 product, and step (e) comprises:

(ea) providing conditions permitting catalytic activity of said enzyme to yield formation of said insoluble reaction product; and

(eb) detecting the presence of said insoluble reaction product on said sensing interface.

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13. The method of any one of Claims 7-11, wherein said signal-amplifying agent comprises a moiety or a particle which directly increases the mass immobilized on the sensing surface, the method comprises in step (e):

5 (ea) detecting the presence of said moiety or particle on said sensing interface.

14. The method of Claim 13, wherein said moiety or particle is a molecule, a super molecular structure or a particle.

15. A method of Claim 14, wherein said particle is a liposome.

16. A system for detecting a target oligonucleotide in a sample, comprising:

10 (i) a sensor device having a sensing interface carrying capturing oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a first portion of the target oligonucleotides;

(ii) verification oligonucleotides having each a nucleotide sequence complementary in at least a portion thereof to a second portion of the target
15 oligonucleotide, other than said first portion; and

(iii) a combination comprising one or both of apparatus and reagents for detecting a verification oligonucleotide bound to the sensing interface.

17. The system of Claim 16, wherein said sensor device is an electrochemical electrode carrying said sensing surface.

20 18. The system of Claim 16 or 17, wherein said apparatus is adapted for the performance of an electrochemical measurement.

19. The system of Claim 16, wherein said sensor device comprises a microbalance quartz-crystal probe carrying the sensing interface.

20. The system according to Claim 20, wherein said detection is based on a
25 microgravimetric quartz-crystal microbalance (QCM) analysis.

21. The system of Claims 16-20, wherein said capturing oligonucleotide has a nucleotide sequence complementary to said first portion which has a length of about 12 nucleotides.

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22. The system of any one of Claims 16-21, wherein the verification oligonucleotide is conjugated to a recognition agent which specifically binds to a signal-amplifying agent.

23. The system of Claim 22, wherein said recognition agent is biotin and said
5 signal-amplifying agent comprises avidin.

24. The system of any one of Claims 16-21, wherein the verification oligonucleotide is conjugated or complexed with a signal-amplifying agent.

25. A system of any one of Claims 16-21, wherein the verification oligonucleotide is conjugated to a first recognition agent, which specifically binds to
10 a recognition partner, the recognition partner being capable of binding also to a second recognition agent, being the same or different from said first recognition agent; the system further comprises a signal amplifying agent comprising a second recognition agent.

26. A system of Claim 24 or 25, when said first and said second recognition
15 agents are biotin and where said recognition partner is avidin or streptavidin.

27. A system of any one of Claims 16-26, wherein said signal-amplifying agent comprises an enzyme which catalyzes a reaction yielding an insoluble reaction product.

28. A system of any one of Claims 16-26, wherein said signal-amplifying agent
20 comprises a particle or moiety which directly increases the mass immobilized on the sensing interface.

29. A system according to Claim 28, when said moiety or particle is a liposome.

30. For use in the method of any one of Claims 1-15 or the system of any one of Claims 16-29, a reagent being at least one member of the group consisting of:

- 25 (i) said verification oligonucleotide;
- (ii) an amplifying agent for amplifying the signal resulting in from binding of said verification oligonucleotide to said sensing interface.

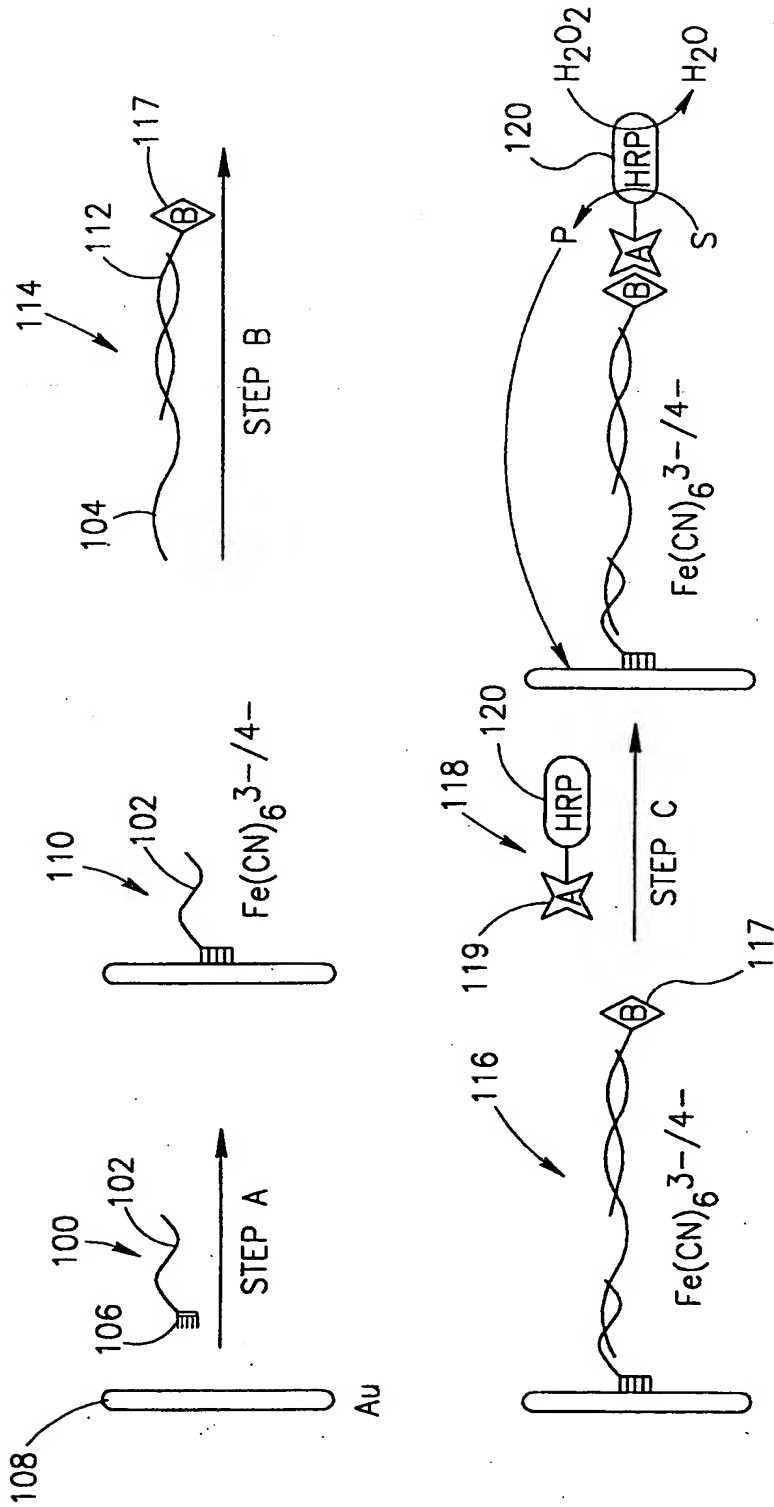


FIG.1

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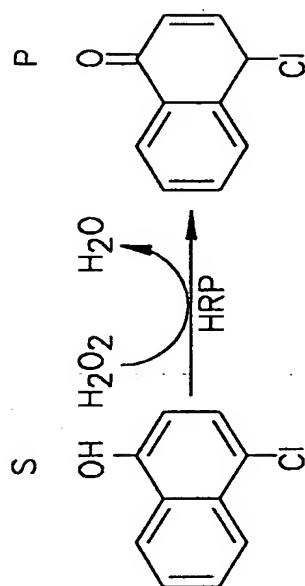


FIG.2B

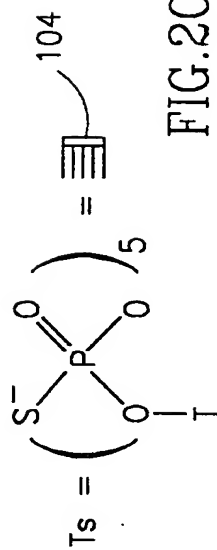


FIG.2C

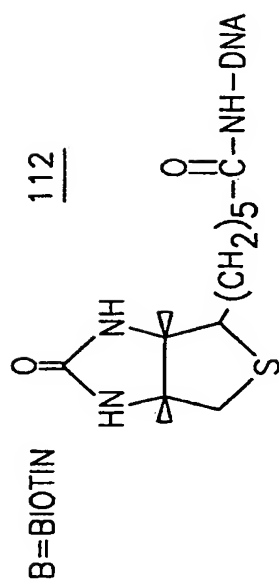


FIG.2A

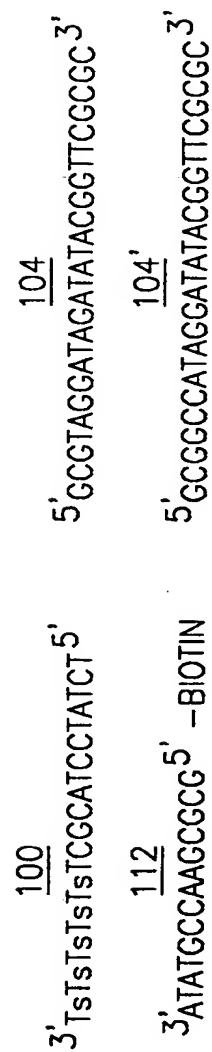


FIG.2D

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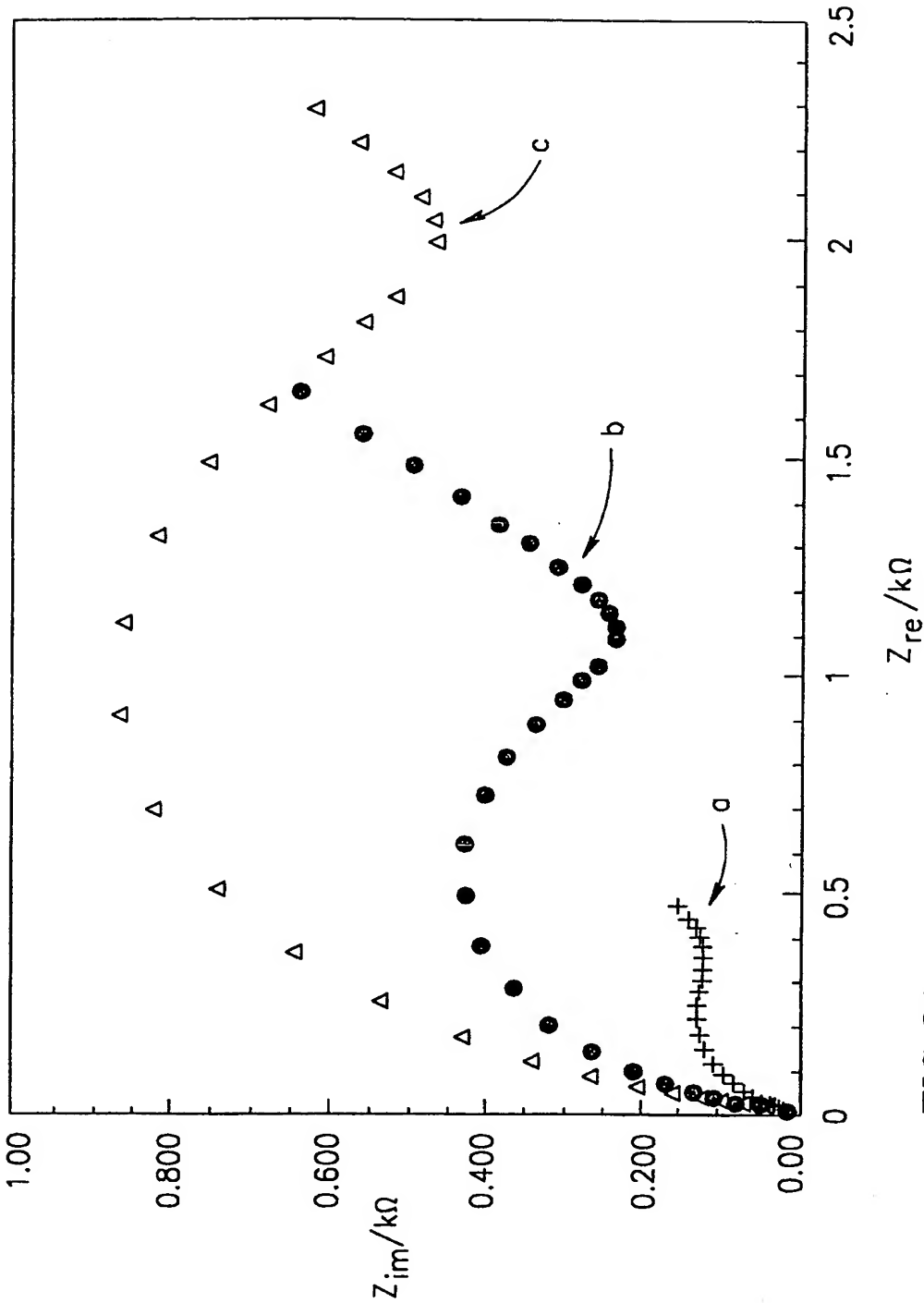


FIG. 3A

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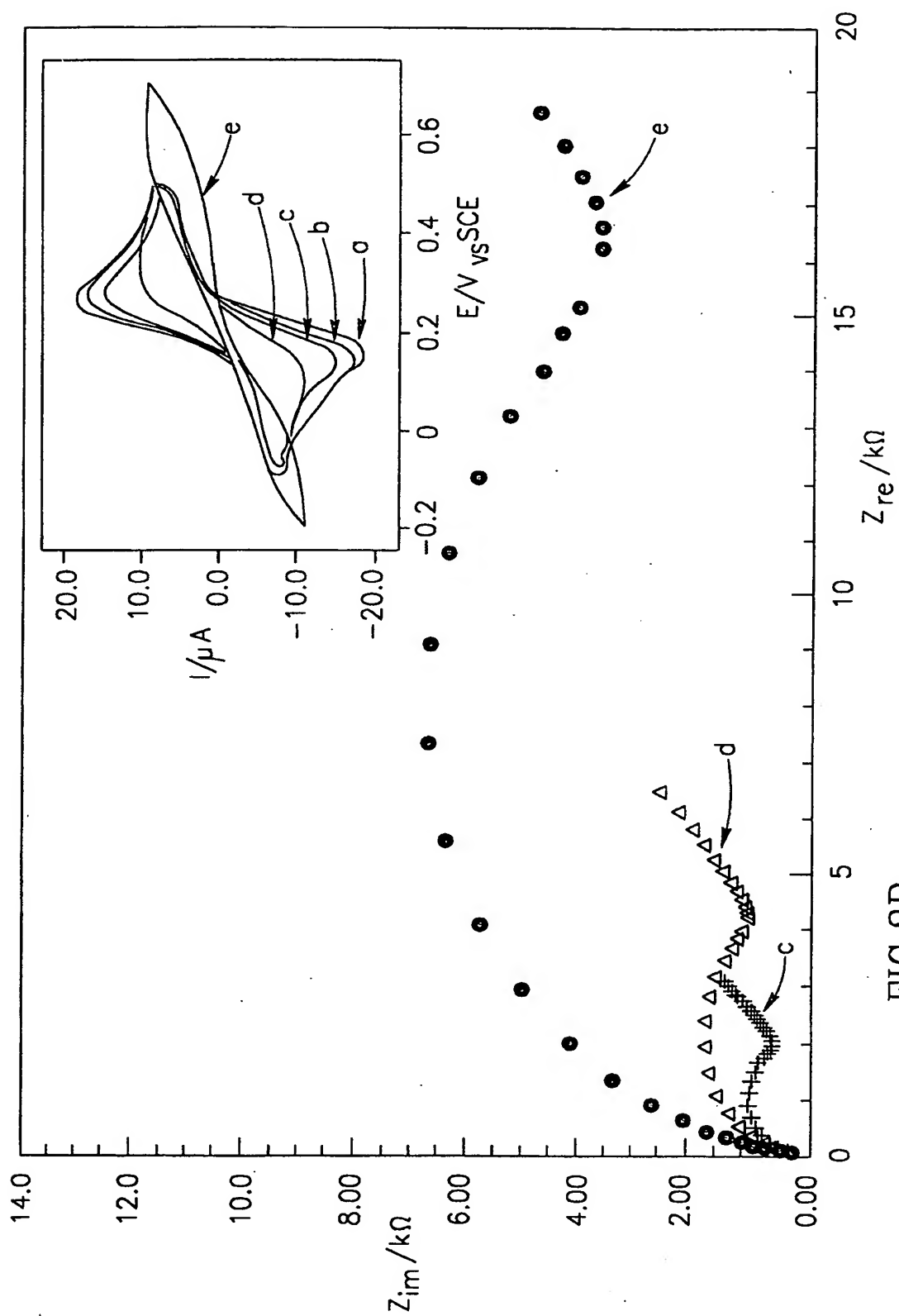


FIG.3B

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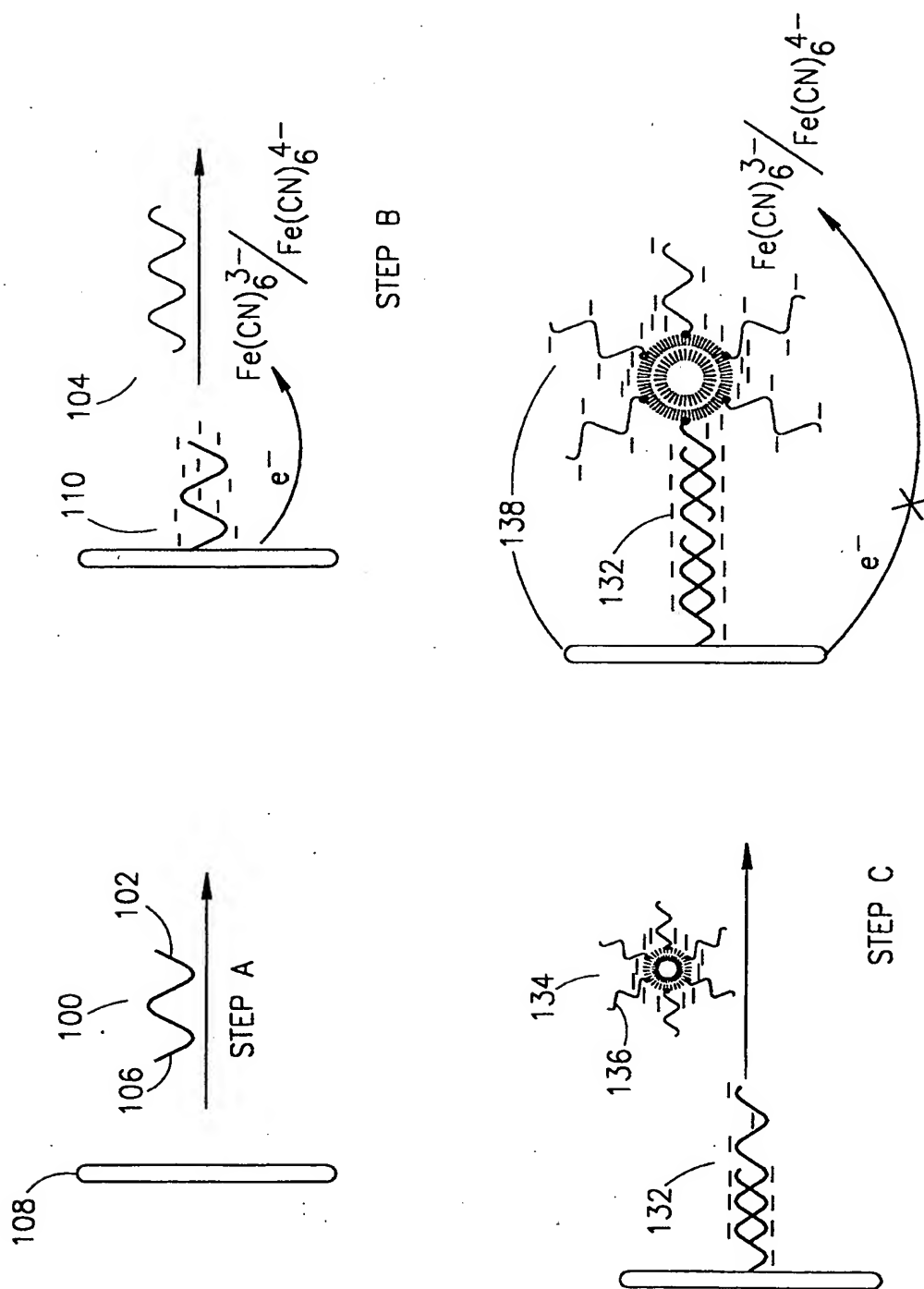


FIG. 4A

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100	5'-TCTATCCTACGCT-(CH ₂) ₆ -SH-3'
104	5'-AGCGTAGGATAGATATACGGTTCGCGC-3'
136	5'-HS-(CH ₂) ₆ -GCGCGAACCGTATA-3'
104'	5'-AGCGCTCCAGTGATATACGGTTCGCGC-3'
136'	5'-biotin-GCGCGAACCGTATA-3'

FIG.4B

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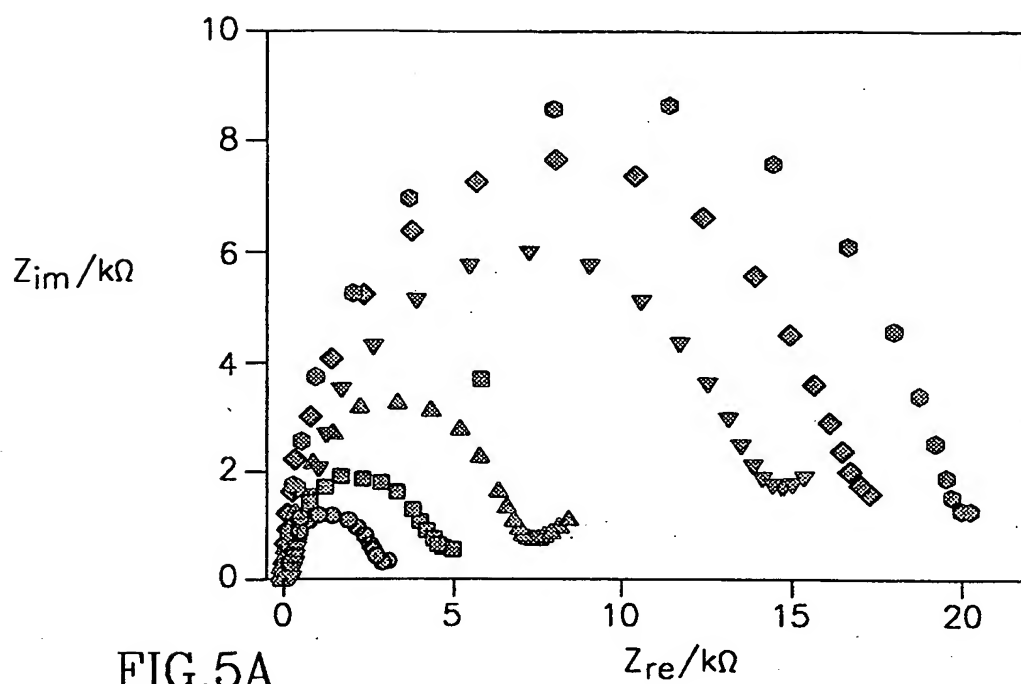


FIG. 5A

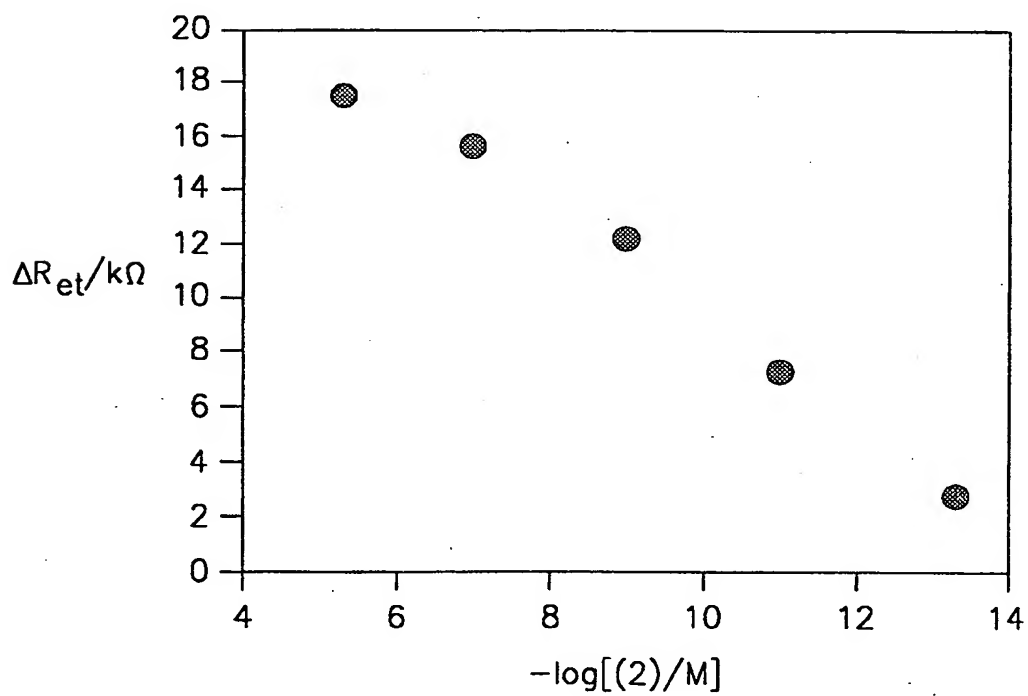
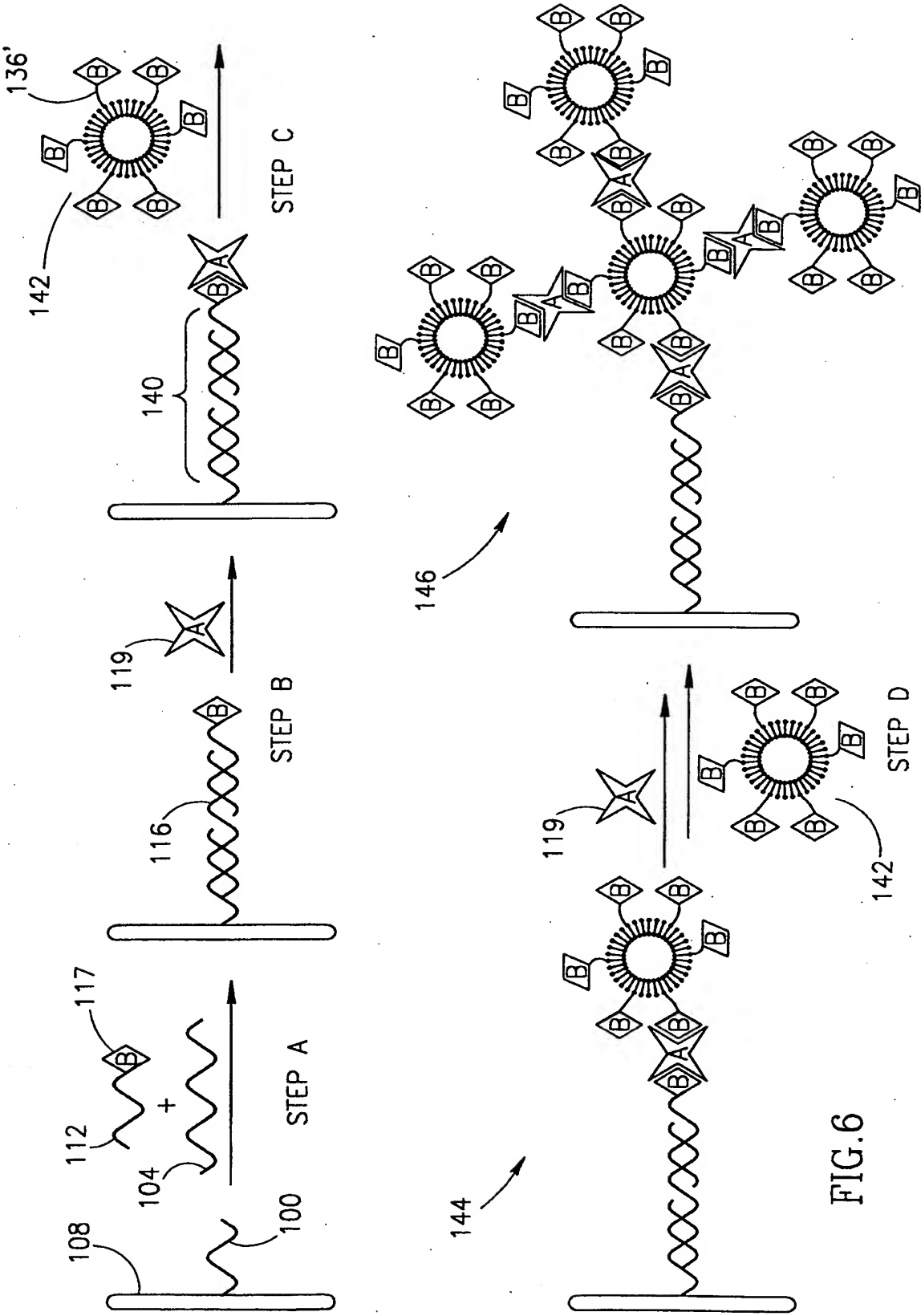
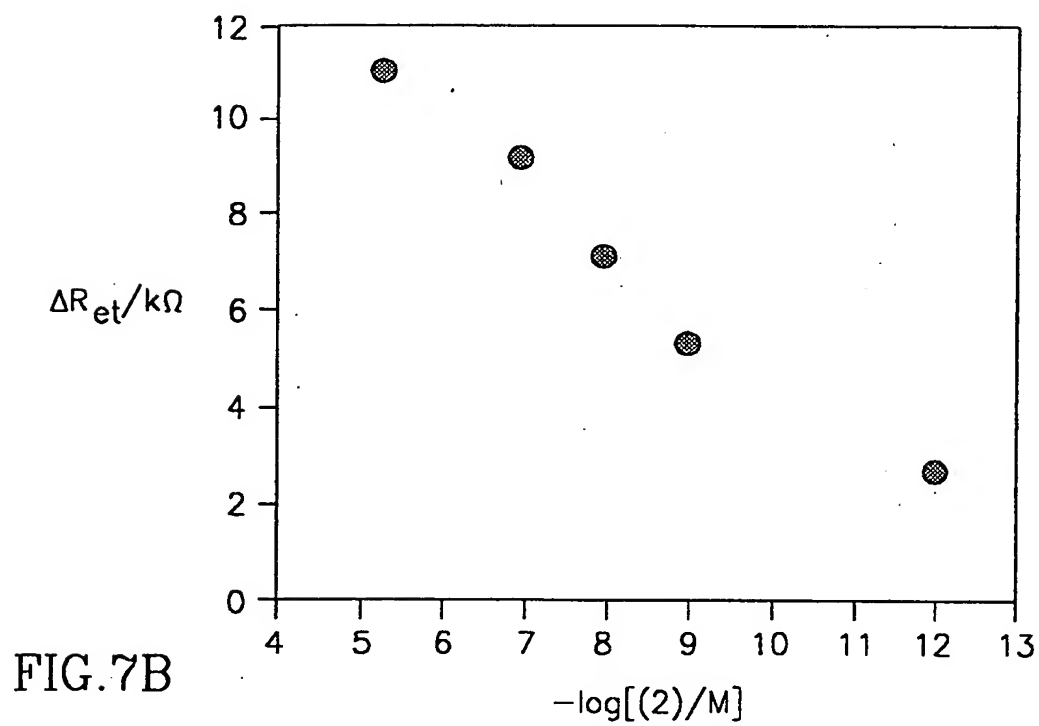
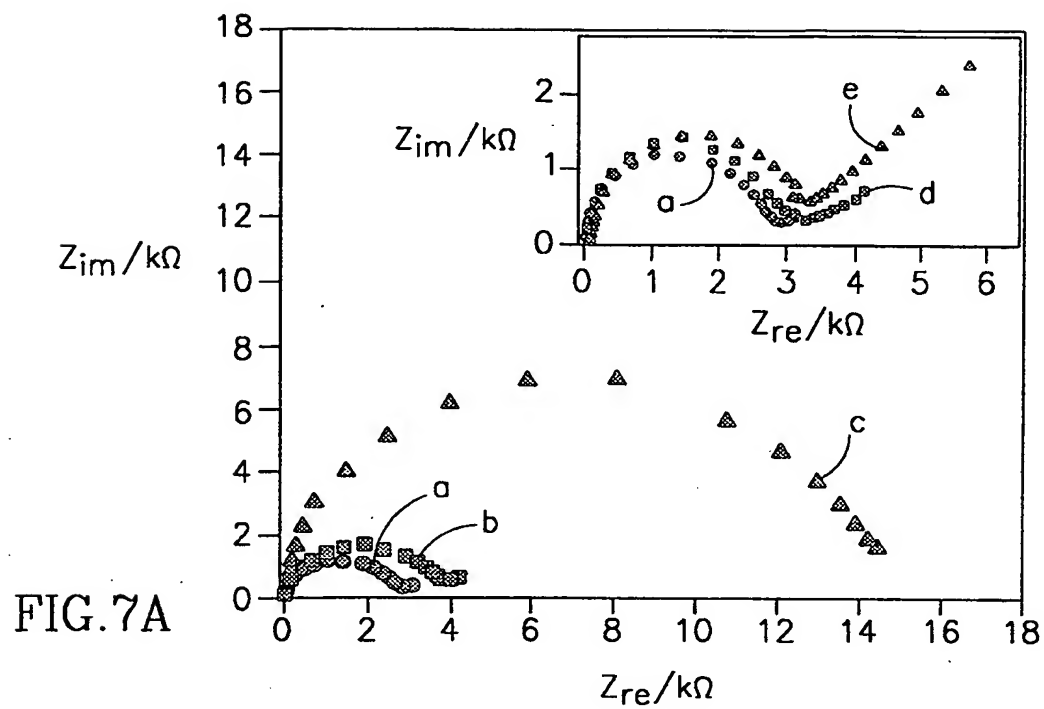


FIG. 5B



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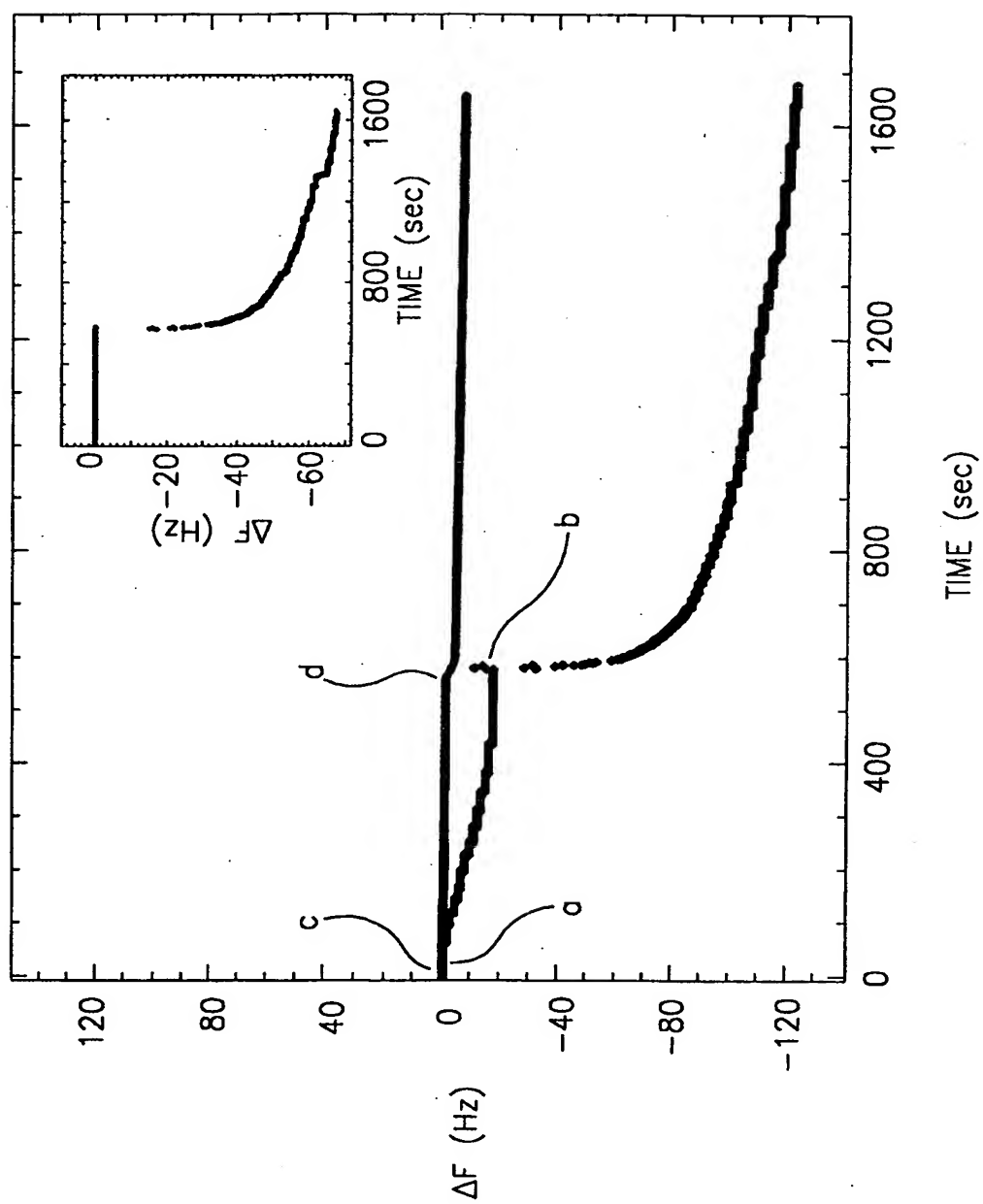


FIG. 8A

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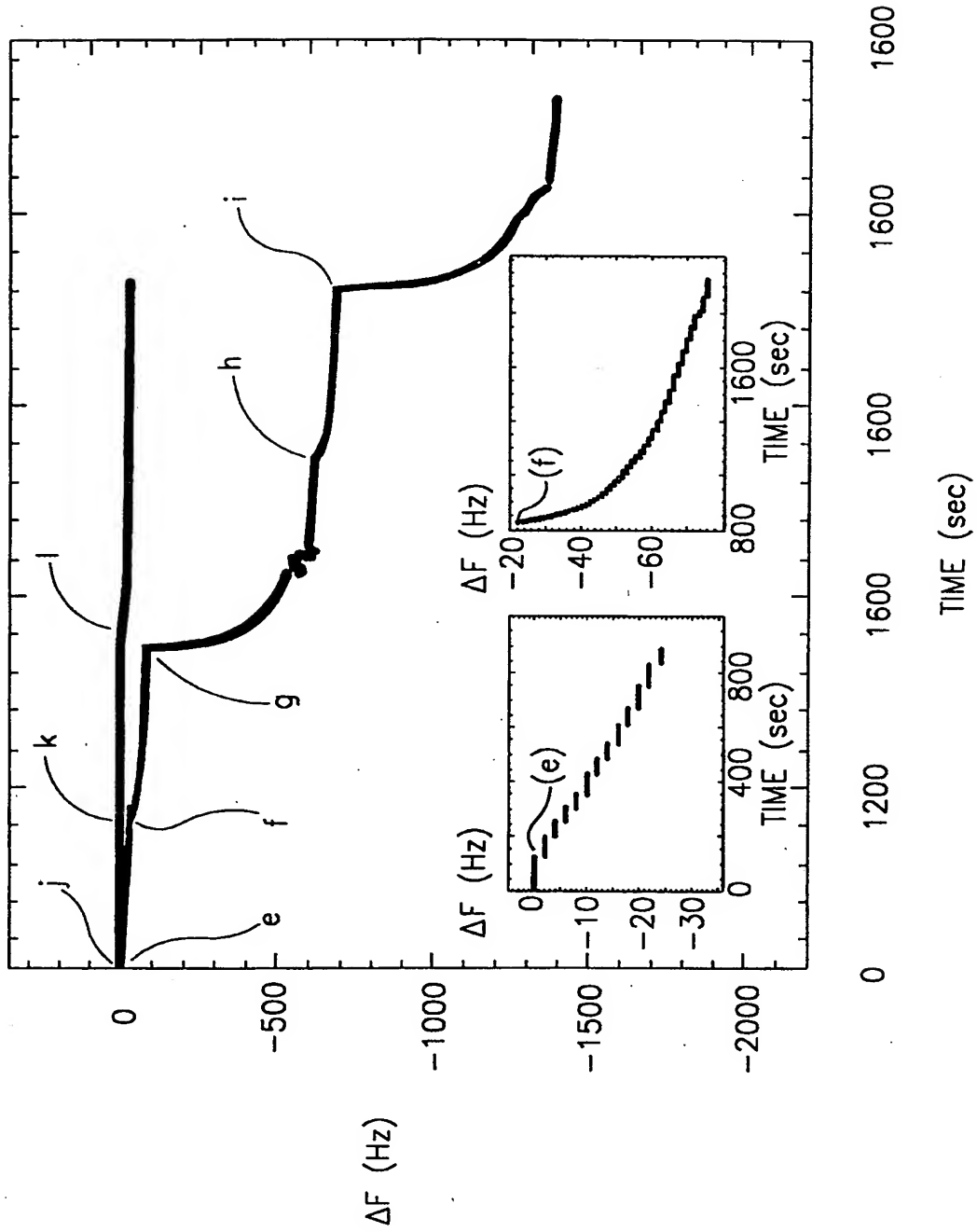


FIG. 8B

SEQUENCE LISTING

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SAMPLE

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<151> 1998-12-01

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WO 00/32813

PCT/IL99/00649

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14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 99/00649

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 695 926 A (CROS PHILIPPE ET AL) 9 December 1997 (1997-12-09) see whole doc esp. claims	1
X	WO 92 08808 A (SISKA DIAGNOSTICS INC) 29 May 1992 (1992-05-29)	1
Y	see claims and figure	2-7, 16
Y	OKAHATA Y. ET AL.,: "Kinetic measurement of DNA hybridization on an oligonucleotide-immobilized 27-MHz quartz crystal microbalance" ANAL. CHEM., vol. 70, - 1 April 1998 (1998-04-01) pages 1288-1296, XP000891733 the whole document	2-7, 16
-/-		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

3 April 2000

Date of mailing of the international search report

13/04/2000

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Authorized officer

Müller, F

INTERNATIONAL SEARCH REPORT

Intern. Patent Application No
PCT/IL 99/00649

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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